Applied and Environmental Microbiology, Apr. 2009, p. 1916–1921 Vol. 75, No. 7
0099-2240/09/$08.00+0 doi:10.1128/AEM.02677-08

Interactions between Coexisting Intracellular Genomes: Mitochondrial Density and *Wolbachia* Infection▽

L. Mouton,* H. Henri, and F. Fleury

Université de Lyon, Université Lyon 1, Laboratoire de Biométrie et Biologie Evolutive, UMR CNRS 5558, 43 Boulevard du 11 Novembre 1918, 69622 Villeurbanne Cedex, France

Received 24 November 2008/Accepted 22 January 2009

Many arthropods are infected with maternally transmitted microorganisms, leading to the coexistence of several intracellular genomes within the host cells, including their own mitochondria. As these genomes are cotransmitted, their patterns of evolution have been intimately linked, with possible consequences for the diversity and evolution of the host mitochondrial DNA. The evolutionary aspects of the situation have been thoroughly investigated, especially the selective sweep on the mitochondria as a result of *Wolbachia* invasion, whereas direct interactions between mitochondria and intracellular symbionts within the host cells or body have received little attention. Since endosymbionts exploit host resources but mitochondria supply energy to meet the bioenergetic demands of organisms, an unanswered question concerns the correlation between their densities. Here, we investigated the influence of *Wolbachia* symbiosis on mitochondrial density in two parasitic wasps of *Drosophila* species, both of which are naturally infected by three *Wolbachia* strains, but they differ in their degree of dependency on these bacteria. In *Leptopilina heterotoma*, all *Wolbachia* strains are facultative, whereas *Asobara tabida* requires a strain of *Wolbachia* for oogenesis to occur. In both species, *Wolbachia* infections are stable and well regulated, since the density of each strain does not depend on the presence or absence of other strains. Using lines that harbor various *Wolbachia* infection statuses, we found that mitochondrial density was not affected by the infection regardless of the sex and age of the host, which is strongly reminiscent of the independent regulation of specific *Wolbachia* strains and suggest that the protagonists coexist independently of each other as the result of a long-term coevolutionary interaction.

Symbiotic interactions with cytoplasmic maternally transmitted microorganisms (endosymbionts) are widespread in arthropods, which leads to the coexistence of several genomes within the host cells that may have direct or indirect effects on the host's biology/physiology (10, 26, 28, 31, 41). These interacting genomes form an entity, known as the symbiome (30), that includes the host genome, the mitochondria, and a number of endosymbionts, mainly bacteria (including several different species or strains in multiple infections). As they share the same intracellular host environment, interactions among the integrated elements (symbionts and mitochondria) could occur, including interference with their respective intrinsic multiplication rates and relative densities, competitive exclusion, cooperation, or synergism.

The influence of vertically transmitted microorganisms on mitochondrial DNA diversity and evolution have often been studied, especially in the case of bacteria that manipulate host reproduction, such as *Wolbachia*, but few studies have investigated the relationships between bacterial symbiosis and mitochondrial density. Nevertheless, whereas mitochondria, called the cellular power plants, supply energy to meet the host's energetic demands, endosymbionts exploit host resources, and so they are costly in terms of energy (7) even if in some cases the cost-benefit balance brings some advantage to the host individual (for a review, see reference 22). Since the density of mitochondria may be increased in response to the higher bioenergetic demands of organisms (21), infection by endosymbionts could affect the number of mitochondria. On the other hand, when associations are stable with a long coevolutionary history, and as the maternally inherited symbionts and mitochondria are cotransmitted, they may coexist without any consequences for their relative numbers.

Wolbachia species are widespread bacteria that induce various changes in their hosts' reproduction, which allows them to increase the proportion of infected females (34), thus spreading the bacterium within the host population (36). During invasion, the cotransmission of symbionts and mitochondria leads these bacteria to have an indirect impact on the DNA diversity of the mitochondria (14, 35, 37) as a result of a selective sweep of the mitotype associated with the infection (13). However, few studies have focused on the mitochondrial quantity in invertebrates (1, 17, 18), and as far as we are aware, no studies have focused on the interactions between facultative symbionts and mitochondria at the level of the individual host organism. Only a few examples are available on the effect of obligatory symbionts (known as primary symbionts) on mitochondrial energy metabolism. For example, Heddi et al. (11) showed that the weevil primary symbiont SOPE (*Sitophilus oryzae* principal endosymbiont) enhanced the enzymatic activity of the mitochondria, but nothing is known about the consequence of the presence of the secondary symbiont, a *Wolbachia* species, on the mitochondrial compartment of this species.

In the study reported here, we investigated how the cohabitation between mitochondria and bacterial endosymbionts works by determining the influence of *Wolbachia* infection on mitochondrial density using quantitative PCR in two parasitic

* Corresponding author. Mailing address: Laboratoire de Biométrie et Biologie Evolutive (UMR-CNRS 5558), Université Claude Bernard, Lyon 1.43, Bd. du 11 Novembre 1918, 69622 Villeurbanne Cedex, France. Phone: 33 (0)4 72 43 29 08. Fax: 33 (0)4 78 89 27 19. E-mail: mouton@biomserv.univ-lyon1.fr.

▽ Published ahead of print on 30 January 2009.

wasps of *Drosophila* spp., *Leptopilina heterotoma* and *Asobara tabida*. In these wasp species, all host individuals are naturally infected by three *Wolbachia* strains and incur a moderate cost of infection (8, 24). Moreover, *Wolbachia* strains are specifically regulated, since their densities are independent of the presence of other strains within the same host, which suggests that there is little or no competition between the different strains (23, 24). However, these host species differ in their relationships with and dependency on their bacteria. The three *Wolbachia* strains that infect *L. heterotoma* all are facultative and alter host reproduction by cytoplasmic incompatibility (34), whereas *A. tabida* harbors two facultative strains that induce cytoplasmic incompatibility and another strain (*w*Atab3) that is required for host oogenesis to occur (5). In *A. tabida*, therefore, there are different degrees of the integration of *Wolbachia* species within the same host individual.

In this particular context of the regulation and stability of symbiosis, we investigated the effect of *Wolbachia* infection on the mitochondrial compartment. In these two wasp species, we compared mitochondrial density among lines sharing the same nuclear genetic background but with differing infection statuses. In *L. heterotoma*, one line infected by three strains of *Wolbachia* was compared to a derived uninfected line, and in *A. tabida*, one line infected by three strains of *Wolbachia* was compared to a derived line infected only with the obligatory strain *w*Atab3. The intracellular symbiotic compartments of these lines had been manipulated, which made it possible to investigate the consequences of infection on host mitochondrial density. As it had previously been shown that the mitochondrial DNA copy number may vary with sex and age (2), measures were done on males and females at 1 and 5 days of age.

MATERIALS AND METHODS

Insect strains and rearing. The two hymenopteran parasitoids of *Drosophila* species, *Leptopilina heterotoma* (Figitidae) and *Asobara tabida* (Braconidae), used in the study both are naturally infected by three *Wolbachia* strains (39). The wasps were reared on a standard diet, at 20°C with a 12-h light/12-h dark cycle and 70% relative humidity, without larval or adult competition by providing four mated females with 150 individuals of the *Wolbachia* species-free strain of *Drosophila melanogaster* larvae originating from Lyon (France).

To investigate the effects of age and sex on mitochondrial density, quantifications were performed on 1-day-old and 5-day-old males and virgin females. The physiological conditions were carefully controlled by keeping the newly emerged individuals for 5 days, during which they were supplied with water and honey.

We used an inbred line of *Leptopilina heterotoma* A7(123) that originated from Antibes (France) and which naturally harbors three *Wolbachia* species strains, *w*Lhet1, *w*Lhet2, and *w*Lhet3 (39). The uninfected line A7(0) had been derived from the A7(123) line by antibiotic treatments many generations before the experiment, thus making it possible to analyze individuals with the same genetic nuclear background (38).

Asobara tabida organisms are naturally infected by three *Wolbachia* strains, *w*Atab1, *w*Atab2, and *w*Atab3 (39). The triply infected line Pi(123) is an inbred line originating from Pierrefeu (France). A derived line infected only with the strain *w*Atab3 had been obtained by means of moderate antibiotic treatment a number of generations ago (F. Dedeine, personal communication). *w*Atab3 is required for oogenesis in *A. tabida* (5) and, thus, cannot be eliminated from this species, providing an opportunity to analyze the relationship between *Wolbachia* and mitochondrial density in a more integrated association.

Molecular methods. (i) DNA extraction. Total DNA was extracted from single individuals. The insects were frozen at −80°C and homogenized using 5-mm steel beads in a tissue lyser (Qiagen) at 15 Hz for 20 s. DNA was extracted using the Nucleospin tissue kit (Macherey-Nagel) according to the manufacturer's instructions. The final elution volume was 100 μl.

(ii) PCR. The infection statuses of the insect lines were checked before the experiment by amplifying the *Wolbachia* surface protein (*wsp*) gene using specific primers for each *Wolbachia* strain. This standard procedure is described in Vavre et al. (39). Results showed a homogeneous infection status for the 10 individuals tested per line.

(iii) Quantitative PCR. Mitochondrial and whole *Wolbachia* species densities were measured using the number of copies of the *COI* genes (cytochrome *c* oxidase subunit I) and the *wsp* gene, respectively, divided by the number of copies of the nuclear *18SrRNA* gene, which was used as a housekeeping gene. Quantifications were done individually on 10 insects per modality. The mitochondrial quantity is thought to be directly correlative with the number of *COI* gene copies (1). The primers for the *COI* gene of *L. heterotoma* were A7-COI174 (5′-GACATCCTGGGGTTTCAACT-3′) and A7-COI380 (5′-TTCCTCCTGCT AAAACAGGTAA-3′), and those used for *A. tabida* were Pi-COI24 (5′-GGG GCTCCAGATATAGCTTTC-3′) and Pi-COI233 (5′-TGAAGATGCTCCAGC TAAATG-3′). These primers had been derived from the universal *COI* gene primers described in Simon et al. (32). The corresponding amplicons were 207 and 210 bp in size, respectively. We checked that COI primers do not amplify any part of the *Wolbachia* genome by in silico PCR against the three complete *Wolbachia* genomes available (http://insilico.ehu.es/PCR/) (3) and PCR realized on *Drosophila* species harboring closely related *Wolbachia* strains, such as *L. heterotoma* and *A. tabida* (39). The total number of *Wolbachia* cells was determined in all of the infected individuals, i.e., A7(123) individuals for *L. heterotoma* and Pi(3) and Pi(123) individuals for *A. tabida*, by using the generalist primers 81F/691R to amplify all *Wolbachia* strains as described in Mouton et al. (24). These primers amplified only the single-copy *wsp* gene and not the two paralogous genes that have been reported for the *w*Mel strain genome (40). The primers used to amplify the nuclear gene *18SrRNA* (a multicopy gene) were 18s.lo1 and NS58+2 (29). Measurements were performed on the LightCycler 480 real-time PCR system (Roche). The 10-μl reaction mix contained 200 nM of each primer, 5 μl of LightCycler 480 SYBR green I master mix (Roche), and 1 μl of template DNA. For the *COI* and *18SrRNA* genes, the amplifications consisted of 10 min at 95°C followed by 35 cycles, each consisting of denaturing for 10 s at 95°C, annealing for 10 s at 60°C, and elongation for 10 s at 72°C.

Statistical analysis. Statistical analyses were performed on the ratios calculated from the gene copy numbers of *COI* and *wsp* divided by the nuclear gene *18SrRNA* copy number. Since *L. heterotoma* and *A. tabida* are haplo-diploid species, i.e., the males have *n* chromosomes while females have 2*n* chromosomes, the ratios were multiplied by two for the females in order to obtain the ratios per haploid genome. Results were analyzed using R statistical software (http://www.R-project.org) after arc-sin-square root transformation. Data normality and homoscedasticity were verified by Shapiro and Bartlett tests, respectively. For mitochondrial data, analyses were done by two-way analysis of variance (ANOVA2) on the two sexes separately, because variance was not the same in both sexes (the factors were infection status and age). Comparisons including both males and females were performed using Mann-Whitney nonparametric tests.

RESULTS

Influence of sex and age. We studied the influence of sex and age on mitochondrial quantity by determining the ratio of mitochondrial DNA/insect nuclear gene copies per haploid genome in males and females of 1-day- and 5-day-old *L. heterotoma* and *A. tabida* organisms. In both species we observed that this ratio is less variable and lower in males than in females (Fig. 1 and 2).

In *L. heterotoma* (Fig. 1), males have almost half of the female mitochondrial DNA content, and females have more mitochondria at emergence than at 5 days later. The relative *Wolbachia* density does not vary with age, and females have more *Wolbachia* strains than males, as previously observed (23). The mitochondrion/*Wolbachia* density ratios range from 3:1 to 5:1 in females and 7:1 to 8:1 in males.

In *A. tabida* (Fig. 2), females harbor on average seven times more mitochondrial DNA copies/haploid genome than males. *Wolbachia* density is higher in females than in males and is higher in triply infected than in singly infected females ($P < 0.0001$ by ANOVA) (Fig. 2), which is consistent with data reported previously (24). The mitochondria/*Wolbachia* density ratio is higher in the Pi(3) line than in the Pi(123) line. In triply

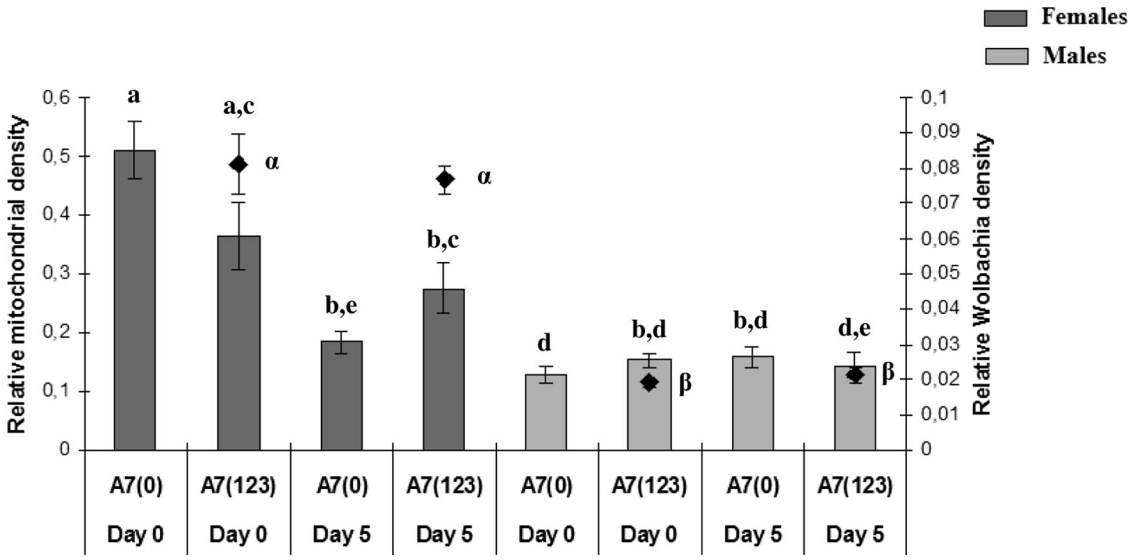

FIG. 1. Relative mitochondrial density (histogram) and *Wolbachia* density (diamond) in *Leptopilina heterotoma*. These densities were estimated by the number of *COI* gene (cytochrome *c* oxidase subunit 1) copies and the number of *wsp* (*Wolbachia* surface protein) copies, respectively, relative to one copy of the nuclear gene *18SrRNA* per haploid genome. The measures were done on 1-day-old and 5-day-old males and females, which were infected by three *Wolbachia* strains [line A7(123)] or were left uninfected [line A7(0)]. Values correspond to the average results for 10 individuals per experimental condition. Bars show the standard errors. Means of mitochondrial/*Wolbachia* densities marked with the same Latin letters/Greek letters, respectively, are not significantly different ($P = 0.05$ by a Mann-Whitney test).

infected individuals, this ratio is around 1 in males and 2 in females, which is lower than the ratios observed for *L. heterotoma*. This difference could be related to the obligatory status of *Wolbachia* in *A. tabida* (5).

Influence of *Wolbachia* infection on mitochondrial density. The number of mitochondria is not statistically different between infected and uninfected individuals in *L. heterotoma* (Fig. 1) ($P > 0.5$ for females and males, as determined by ANOVA2), or between the Pi(3) and Pi(123) lines in *A. tabida* (Fig. 2) ($P > 0.05$ for both sexes, as determined by ANOVA2), despite the higher density of *Wolbachia* in triply infected individuals ($P < 0.0001$ as determined by ANOVA2). These findings indicate that infection by *Wolbachia* species does not affect the mitochondrial density.

DISCUSSION

The aim of this study was to investigate, in two insect species that had established obligatory or facultative associations with symbiotic bacteria, the possible interference between mitochondria and endosymbionts at the level of the individual host.

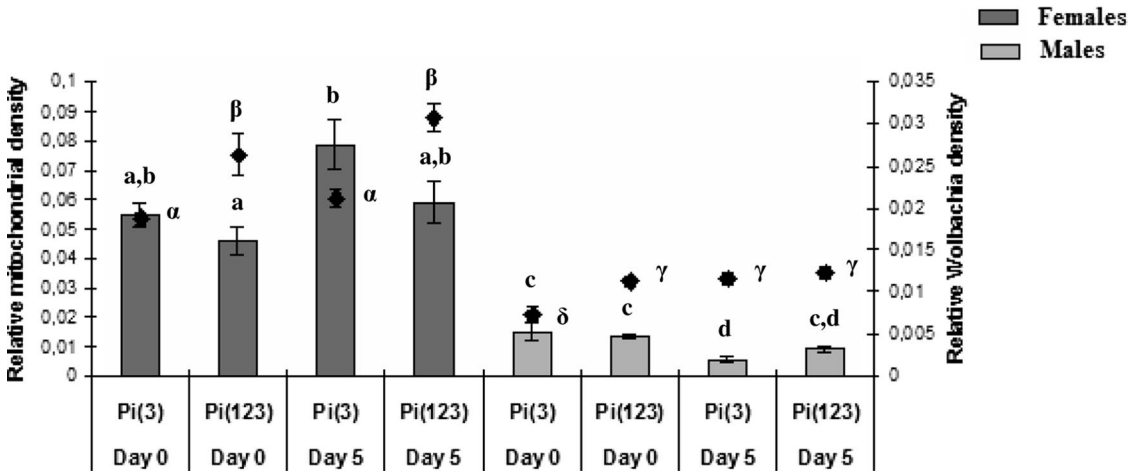

FIG. 2. Relative mitochondrial density (histogram) and *Wolbachia* density (diamond) in *Asobara tabida*. These densities were estimated by the number of *COI* gene (cytochrome *c* oxidase subunit 1) copies and the number of *wsp* (*Wolbachia* surface protein) copies, respectively, relative to one copy of the nuclear gene *18SrRNA* per haploid genome. The measures were done on 1-day-old and 5-day-old males and females, which were infected by the *Wolbachia* strain *w*Atab3 [line Pi(3)]or by three *Wolbachia* species strains *w*Atab1, *w*Atab2, and *w*Atab3 [line Pi(123)]. Values correspond to the average results for 10 individuals per experimental condition. Bars show standard errors. Means of mitochondrial/*Wolbachia* densities marked with the same Latin letters/Greek letters, respectively, are not significantly different ($P = 0.05$ by a Mann-Whitney test).

We tested whether the presence of symbionts, more precisely that of *Wolbachia*, influences host mitochondrial density. Measures were done on 1-day- and 5-day-old males and females to determine the influence of sex and age. In both *A. tabida* and *L. heterotoma*, females are diploid and males are haploid, so the results have been expressed as the DNA copy number per haploid genome.

To the best of our knowledge, the only study to have referred to the correlation between mitochondrial density and *Wolbachia* infection is that published by Ballard and Melvin (1). However, the main focus of their study was to investigate the influence of tetracycline treatment on mitochondrial density in *Drosophila simulans* regardless of whether they were infected with *Wolbachia*. The data presented indicated that the level of mitochondrial DNA density depends more on the host line than on *Wolbachia* infection, so in the present study we controlled the genetic background of the host.

We observed that the females had a higher mitochondrial density than the males, i.e., two and seven times higher in *L. heterotoma* and *A. tabida*, respectively. This phenomenon is well known in mammals, and a recent paper by Ballard et al. (2) showed that it also is true in *Drosophila*. This difference between the sexes may be linked to the energy requirements of reproduction, which may be higher in females than in males (2).

Differences in mitochondrial density between 1-day- and 5-day-old individuals depend to a great extent on the sex and host species. Mitochondrial density is fairly constant in the males of both species. The only difference seen was in *A. tabida* males, which displayed a decrease in mitochondrial density with age. The mitochondrial density was more variable in the females: in *L. heterotoma* there was a trend toward a decrease in mitochondrial density with age, whereas the opposite trend was observed in *A. tabida*. A decrease in the proportion of mitochondrial to total DNA already had been reported to occur in *Drosophila melanogaster* after the first week of life (17). At present, the mechanisms involved in controlling the mitochondrial number are unknown (21), but it has been shown that many factors can affect the level of variation with age (18), and this could explain the interspecific variability observed in the present study. For example, the timing of mitochondrial maturation is variable between species. However, whereas mitochondrial maturation is known to be completed after 9 days in *Drosophila* (33), there are no data available for parasitic wasps. Interspecies variability also could be due to the oogenesis process, which may be different between the two species. Most oocytes of *L. heterotoma* females already are mature when the female emerges (i.e., it is a proovogenic species), whereas in *A. tabida* some of the eggs continue to mature after the emergence (i.e., it is a prosynovogenic species) (F. Fleury, personal communication). Since oocyte maturation increases the energy requirement, this could explain why, in females, the mitochondrial density increases during the first 5 days after emergence in *A. tabida*, whereas it decreases in *L. heterotoma*.

The mitochondrial density relative to the nuclear haploid genome differs greatly between the two wasp species: the range is 0.05 to 0.08 in *A. tabida* and 0.2 to 0.5 in *L. heterotoma*. These values correspond to the ratio of the copy numbers of the *COI* and *18SrRNA* genes and do not correspond to the number of mitochondrial molecules per cell. Indeed, the *18SrRNA* gene is a multicopy gene, and the number of copies per haploid genome varies considerably between species. For example, in two insect species, the fly *Sabethes cyaneus* and the mosquito *Aedes flavopictus*, the numbers of copies of the *18SrRNA* genes per genome have been estimated to be around 40 and 1,000, respectively (15). Another insect, *Locusta migratory*, harbors up to 4,000 copies in some tissues (25). As we have no information about the *18SrRNA* gene copy number in the genomes of *A. tabida* and *L. heterotoma*, we were unable to determine the number of mitochondria per cell in these two species (in mammals, most cells in culture have 1,000 to 5,000 mitochondria, but we have no data for insects [21]), and so we cannot compare their relative mitochondrial densities. For similar reasons, it also is impossible to compare the relative density of *Wolbachia* in the two parasitoid species.

In triply infected individuals, the mitochondria/*Wolbachia* ratio is higher for *L. heterotoma* (7 to 8 in males, 4 in females) than *A. tabida* (around 2 in females, 1 in males), which can be explained by the differing levels of *Wolbachia* dependency in these two species. It is well known that *Wolbachia* species are not uniformly localized throughout the host body but are found mainly in germ line tissues, which favors its transmission (6). It would be interesting to know if the number of mitochondria per cell differs depending on whether *Wolbachia* is present within this cell or not and if the mitochondria/*Wolbachia* ratio varies between the host cells and tissues. Indeed, when the mitochondria and *Wolbachia* occupy the same cells, they could compete for space.

A recent study has shown that the presence of *Wolbachia* can induce a higher production of reactive oxygen species (ROS) in cell lines of the mosquito *Aedes albopictus* (4). ROS-dependent immunity is one of the first responses to microbial infection (12). A high concentration of ROS generates oxidative stress, which can cause alterations of mitochondria, and the mitochondrial DNA copy number may be increased to compensate for the damage caused (for a review, see reference 16). This clearly raises the question of whether the presence of *Wolbachia*, which is costly in terms of energy and induces oxidative stress (4), leads to an increase in mitochondrial quantity. We have no idea about the influence of *Wolbachia* infection on ROS in *L. heterotoma* and *A. tabida*. However, in these species, even if the presence of *Wolbachia* does lead to an increase of ROS production, it clearly does not affect the mitochondrial density, since our results indicated that there was no difference in mitochondrial density between individuals of the same sex and age whatever their infection status.

Various hypotheses have been put forward to explain the absence of any correlation between *Wolbachia* infection and mitochondrial density.

First, it is possible that *Wolbachia* requires little energy. In this case, the increase in the energy demand might be low enough to be provided by the number of mitochondria present in uninfected individuals. The energy required could be linked to the cost of *Wolbachia* infection, which varies considerably in different species (8, 9, 20, 24, 27). A moderate cost, such as that induced by *Wolbachia* in *L. heterotoma* and *A. tabida* (8, 24), could involve a low cost in terms of energy. In more costly host-*Wolbachia* associations the results could be different, and *Wolbachia* infection could affect the mitochondrial quantity. For example, the highly virulent strain *popcorn* induces degen-

eration and early death in *Drosophila melanogaster* (20). The modification induced in the host's metabolism could lead to the deregulation of the mitochondrial number. It has been shown that the cost of infection and *Wolbachia* density are positively correlated, with a higher bacterial density leading to an increase in the cost of infection (19, 24). In *L. heterotoma* and *A. tabida*, *Wolbachia* symbiosis is well regulated, since there is a strain-specific regulation of bacterial density (23, 24), which limits the total bacterial load and, thus, the cost of infection.

Another possible explanation is that mitochondria compensate for the higher bioenergetic demands by enhancing the energy they produce in infected individuals without any change in the number of mitochondria. One way this could happen would be that in the presence of *Wolbachia*, mitochondrial enzymatic activities are improved, as has been shown with some primary symbionts (10).

The lack of variation in the mitochondrial density between lines infected by *Wolbachia* or not left uninfected also could be the result of coevolutionary processes. Indeed, it is possible that at the establishment of the association with *A. tabida* and *L. heterotoma*, the infection affected the mitochondrial load but reciprocal selective pressures then led to an equilibrium with an optimal number of mitochondria that now cannot be affected by *Wolbachia* removal. This can result from an evolutionary response from either the host or *Wolbachia* side. One way to test this hypothesis would be the analysis of a recent association, for example, after the injection or horizontal transfer of symbionts, to determine the mitochondrial load change. However, to date, all of the attempts of injection experiments using *A. tabida* and *L. heterotoma* failed. Moreover, injecting bacteria into the host also would have some consequence on its physiology, and it would be difficult to discern the direct influence of *Wolbachia* infection on mitochondrial density.

In the symbiosis studied here, the associations are stable and well regulated, and all of the micropartners seem to live together without the presence of other integrated elements having any marked negative impact, which can be seen as an evolutionary success. In this case, mitochondrial density is not affected by infection. Future studies should investigate the functional level and focus on the size and effectiveness of mitochondria.

ACKNOWLEDGMENTS

We thank the DTAMB for their kind permission to use their Light Cycler system.

This study was funded partly by the CNRS (IFR41-UMR5558).

REFERENCES

1. **Ballard, J. W. O., and R. G. Melvin.** 2007. Tetracycline treatment influences mitochondrial metabolism and mtDNA density two generations after treatment in Drosophila. Insect Mol. Biol. **16:**799–802.
2. **Ballard, J. W. O., R. G. Melvin, J. T. Miller, and S. D. Katewa.** 2007. Sex differences in survival and mitochondrial bioenergetics during aging in *Drosophila*. Aging Cell **6:**699–708.
3. **Bikandi, J., R. San Millán, A. Rementeria, and J. Garaizar.** 2004. In silico analysis of complete bacterial genomes: PCR, AFLP-PCR, and endonuclease restriction. Bioinformatics **20:**798–799.
4. **Brennan, L. J., B. A. Keddie, H. R. Braig, and H. L. Harris.** 2008. The endosymbiont *Wolbachia pipientis* induces the expression of host antioxidant proteins in an *Aedes albopictus* cell line. PLoS One **3:**e2083.
5. **Dedeine, F., F. Vavre, F. Fleury, B. Loppin, M. E. Hochberg, and M. Boulétreau.** 2001. Removing symbiotic bacteria specifically inhibits oogenesis in a parasitic wasp. Proc. Natl. Acad. Sci. USA **98:**6247–6252.
6. **Dobson, S. L., K. Bourtzis, H. R. Braig, B. F. Jones, W. Zhou, F. Rouset, and S. L. O'Neill.** 1999. *Wolbachia* infections are distributed throughout insect somatic and germ lines tissues. Insect Biochem. Mol. Biol. **29:**153–160.
7. **Douglas, A. E.** 1994. Symbiotic interactions. Oxford Science Publications. Oxford University Press, Oxford, United Kingdom.
8. **Fleury, F., F. Vavre, N. Ris, P. Fouillet, and M. Boulétreau.** 2000. Physiological cost induced by the maternally-transmitted endosymbiont *Wolbachia* in the *Drosophila* parasitoid *Leptopilina heterotoma*. Parasitology **121:**493–500.
9. **Fry, A. J., and D. M. Rand.** 2002. *Wolbachia* interactions that determine *Drosophila melanogaster* survival. Evolution **56:**1976–1981.
10. **Heddi, A., H. Charles, and C. Khatchadourian.** 2001. Intracellular bacterial symbiosis in the genus *Sitophilus*: the "biological individual" concept revisited. Res. Microbiol. **152:**431–437.
11. **Heddi, A., F. Lefèbvre, and P. Nardon.** 1993. Effect of endocytobiotic bacteria on mitochondrial enzymatic activities in the weevil *Sitophilus oryzae* (Coleoptera: Curculionidae). Insect Biochem. Mol. Biol. **70:**201–208.
12. **Hoffmann, J. A.** 2003. The immune responses of *Drosophila*. Nature **426:**33–38.
13. **Hurst, G. D. D., and F. M. Jiggins.** 2005. Problems with mitochondrial DNA as a marker in population, phylogeographic and phylogenetic studies: the effects of inherited symbionts. Proc. R. Soc. Lond. B **272:**1525–1534.
14. **Johnstone, R., and G. Hurst.** 1996. Maternally inherited male-killing microorganisms may confound interpretation of mitochondrial DNA variability. Biol. J. Linn. Soc. **58:**453–470.
15. **Kumar, A., and K. S. Rai.** 1990. Chromosomal localization and copy number of 18S + 28S ribosomal RNA genes in evolutionarily diverse mosquitoes (Diptera, Culicidae). Hereditas **113:**277–289.
16. **Lee, H.-C., and Y.-H. Wei.** 2005. Mitochondrial biogenesis and mitochondrial DNA maintenance of mammalian cells under oxidative stress. Int. J. Biochem. Cell Biol. **37:**822–834.
17. **Massie, H. R., M. B. David, and M. M. McMahon.** 1975. Loss of mitochondrial DNA with aging in *Drosophila melanogaster*. Gerontology **21:** 231–238.
18. **Massie, H. R., and T. R. Williams.** 1987. Mitochondrial DNA and life span changes in normal and dewinged *Drosophila* at different temperatures. Exp. Gerontol. **22:**139–153.
19. **McGraw, E. A., D. J. Merritt, J. N. Droller, and S. L. O'Neill.** 2002. *Wolbachia* density and virulence attenuation after transfer into a novel host. Proc. Natl. Acad. Sci. USA **99:**2918–2923.
20. **Min, K. T., and S. Benzer.** 1997. *Wolbachia*, normally a symbiont of *Drosophila*, can be virulent, causing degeneration and early death. Proc. Natl. Acad. Sci. USA **94:**10792–10796.
21. **Moraes, C. T.** 2001. What regulates mitochondrial DNA copy number in animal cells? TRENDS Genet. **17:**199–205.
22. **Moran, N. A., J. P. McCutcheon, and A. Nakabachi.** 2008. Genomics and evolution of heritable symbionts. Annu. Rev. Genet. **42:**165–190.
23. **Mouton, L., H. Henri, M. Boulétreau, and F. Vavre.** 2003. Strain-specific regulation of intracellular *Wolbachia* density in multiply infected insects. Mol. Ecol. **12:**3459–3465.
24. **Mouton, L., H. Henri, M. Boulétreau, N. Profizi, and F. Vavre.** 2004. Virulence, multiple infections and regulation of symbiotic population in the *Wolbachia-Asobara tabida* symbiosis. Genetics **168:**181–189.
25. **Oishi, M., J. Locke, and G. R. Wyatt.** 1985. The ribosomal RNA genes of Locustra migratoria: copy number and evidence for underreplication in a poplyploid tissue. Can. J. Biochem. Cell Biol. **63:**1067–1070.
26. **Oliver, K. M., N. A. Moran, and M. S. Hunter.** 2006. Costs and benefits of a superinfection of facultative symbionts in aphids. Proc. R. Soc. Lond. B Biol. Sci. **273:**1273–1280.
27. **Poinsot, D., and H. Merçot.** 1997. *Wolbachia* infection in *Drosophila simulans*: does the female host bear a physiological cost? Evolution **51:** 180–186.
28. **Rio, R. V., Y. N. Wu, and S. Aksoy.** 2006. Dynamics of multiple symbiont density regulation during host development: tsetse fly and its microbial flora. Proc. R. Soc. Lond. B Biol. Sci. **273:**805–814.
29. **Sanchis, A., A. Latorre, F. Gonzalez-Candelas, and J. M. Michelena.** 2000. An 18S rDNA-based molecular phylogeny of aphidiinae (Hymenoptera: braconidae). Mol. Phyl. Evol. **14:**180–194.
30. **Sapp, J.** 2003. Genesis: the evolution of biology. Oxford University Press, Inc., New York, NY.
31. **Scarborough, C. L., J. Ferrari, and H. C. Godfray.** 2005. Aphid protected from pathogen by endosymbiont. Science **310:**1781.
32. **Simon, C., F. Frati, A. Beckenbach, B. Crespi, H. Liu, and P. Flook.** 1994. Evolution, weighting, and phylogenetic utility of mitochondrial gene sequences and a compilation of conserved polymerase chain reaction primers. Ann. Entomol. Soc. Am. **87:**651–701.
33. **Sohal, R. D.** 1975. Mitochondrial changes in flight muscles of normal and flightless *Drosophila melanogaster* with age. J. Morphol. **145:**337–353.
34. **Stouthamer, R. J., J. A. J. Breeuwer, and G. D. D. Hurst.** 1999. *Wolbachia pipientis*: microbial manipulator of arthropod reproduction. Annu. Rev. Microbiol. **53:**71–102.
35. **Turelli, M.** 1994. Evolution of incompatibility-inducing microbes and their hosts. Evolution **48:**1500–1513.

36. **Turelli, M., and A. A. Hoffmann.** 1991. Rapid spread of an inherited incompatibility factor in California *Drosophila*. Nature **353:**440–442.
37. **Turelli, M., A. A. Hoffmann, and S. W. McKechnie.** 1992. Dynamics of cytoplasmic incompatibility and mtDNA variation in natural *Drosophila simulans* populations. Genetics **132:**713–723.
38. **Vavre, F., F. Dedeine, M. Quillon, P. Fouillet, F. Fleury, and M. Boulétreau.** 2001. Within-species diversity of *Wolbachia*-induced cytoplasmic incompatibilities in haplodiploid insects. Evolution **55:**1710–1714.
39. **Vavre, F., F. Fleury, D. Lepetit, P. Fouillet, and M. Boulétreau.** 1999. Phylogenetic evidence for horizontal transmission of *Wolbachia* in host-parasitoid associations. Mol. Biol. Evol. **16:**1711–1723.
40. **Wu, M., L. V. Sun, J. Vamathevan, M. Riegler, R. Deboy, J. C. Broxnlie, E. A. McGraw, W. Martin, C. Esser, N. Ahmadinejad, C. Wiegand, R. Madupu, M. J. Beanan, L. M. Brinkac, S. C. Daugherty, A. S. Durkin, J. F. Kolonay, W. C. Nelson, Y. Mohamoud, P. Lee, K. Berry, M. B. Young, T. Utterback, J. Weidman, W. C. Nierman, I. T. Paulsen, K. E. Nelson, H. Tettelin, S. L. O'Neill, and J. A. Eisen.** 2004. Phylogenomics of the reproductive parasite Wolbachia pipientis wMel: a streamlined genome overrun by mobile genetic elements. PLoS Biol. **2:**E69.
41. **Zchori-Fein, E., and J. K. Brown.** 2002. Diversity of prokaryotes associated with *Bemisia tabaci* (Gennadius) (Hemiptera: Aleyrodidae). Ann. Entomol. Soc. Am. **95:**711–718.